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Antibiotic resistance genes in municipal wastewater treatment systems and receiving waters in
Arctic Canada

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Abstract

Domestic wastewater discharges may adversely impact arctic ecosystems and local indigenous people, who rely on being able to hunt and harvest food from their local environment. Therefore, there is a need to develop efficient wastewater treatment plants (WWTPs), which can be operated in remote communities under extreme climatic conditions. WWTPs have been identified as reservoirs of antibiotic resistance genes (ARGs). The objective of this work was to quantify the presence of nine different ARG markers (*int1*, *sul1*, *sul2*, *tet(O)*, *erm(B)*, *mecA*, *bla_{CTX-M}*, *bla_{TEM}*, and *qnr(S)*) in two passive systems (waste stabilization ponds [WSPs]) and one mechanical filtration plant operating in two smaller and one large community, respectively, in Nunavut, Canada. Measurement of water quality parameters (carbonaceous oxygen demand, ammonia, total suspended solids, *Escherichia coli* and total coliforms) showed that the WWTPs provided only primary treatment. Low levels of the ARGs (2 log copies/mL) were observed in the effluent, demonstrating that bacteria residing in three northern WWTPs harbour ARGs conferring resistance to multiple clinically-relevant classes of antibiotics. Our results indicate that long-term storage in WSPs benefitted removal of organic material and some ARGs. However, one WSP system showed evidence of the enrichment of *sul1*, *sul2*, *mecA*, *tet(O)* and *qnr(S)*. Further research is needed to fully understand if these ARG releases pose a risk to human health, especially in the context of traditional hunting and fishing activities.

Keywords: Waste stabilization ponds, mechanical filtration, arctic communities, antibiotic resistant bacteria, ARG enrichment, quantitative PCR

1. Introduction

Bacterial antibiotic resistance is an immediate public health concern, broadly acknowledged by governments and health agencies around the world (Ashbolt et al., 2013; Laxminarayan et al., 2013; Rahube et al., 2014). Antibiotic misuse and overuse in both the agricultural industry and the health-care sector has led to the selection of antibiotic resistant pathogenic bacteria (ARB), thus creating new clinical challenges to treating previously curable bacterial infections (Finley et al., 2013). Currently, there are no guidelines regarding the presence of antibiotic residues in wastewater, to which they are being introduced by antibiotic manufacturers, through domestic disposal, by medical sectors, and/or through agricultural practices (Finley et al., 2013). Concerns for human health arises if ARB develop in the environment, including in wastewater treatment plants (WWTPs), through the uptake of antibiotic resistance genes (ARGs) mediated by horizontal gene transfer (e.g., plasmids, integrons, transposons, or gene cassettes) (Ashbolt et al., 2013; Qui et al., 2012; Taylor et al., 2011). The prevalence of ARB and ARGs in the environment can increase the overall ARG pool in environmental bacteria, which can facilitate the transfer of resistance into current and emerging pathogens (Czekalshi et al., 2012). Therefore, properly understanding the reservoirs of ARGs and their evolution and persistence within the environment can help us to assess the risk they pose to human and environmental health.

High concentrations of antibiotic residues, ARB and ARGs have been recently reported in clinical, industrial, and municipal wastewater systems (Czekalski et al., 2012; Martinez, 2009; Narciso-da-Rocha et al., 2014; Rahube et al., 2014; Vaz-Moreira et al., 2014; Volkmann et al., 2004). The nutrient rich environment within a WWTP is ideal for microbial growth and may provide conditions that facilitate transfer of resistance genes between microbial communities

found in the wastewater (Amos et al., 2014). Recent improvements to WWTPs around the world have focused on the removal of biologically available nitrogen and phosphorus, but they have not been specifically designed to remove antibiotic residues and ARB (Rahube et al., 2014).

In the Canadian Arctic, waste stabilization ponds (WSPs) are the preferred passive system for treating municipal wastewater, while a few communities utilize mechanical systems (Holeton et al., 2011). Arctic WSPs are operated as controlled discharge storage ponds, where the wastewater is held for an average of 200-250 days followed by the annual decant (emptying of the WSP) at the end of the summer. Most communities do not have piped water and wastewater infrastructure. Instead, individual homes have wastewater storage tanks, and waste is collected using septic haulage trucks and transported to WSPs located on the edge of the communities (Ragush et al., 2015). New regulations for municipal wastewater treatment (Wastewater Systems Effluent Regulations [WSER], Environment Canada, 2015) have been implemented but are not being applied to Canada's northern regions, since arctic communities would have difficulties complying with the regulations in terms of nutrient and contaminant removal (Holeton et al., 2011; Huang et al., 2017; Ragush et al., 2015). While these regulations are in place to mitigate environmental and health risks, these northern communities are faced with several obstacles (e.g., infrastructure, climate, funding, community capacity, lack of suitable design guidelines, etc.) that currently limits their ability to comply with the WSER (Ragush et al., 2015).

Despite a study done by Dalloo et al. (2008) observing the increasing number of community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) cases in northern communities, knowledge about the occurrence and removal of ARGs in arctic Canadian municipal wastewater, WWTP effluents and local receiving waters is limited. So far only one

study has reported on the presence of tetracycline and sulfonamide resistance genes in the WSP-wetland WWTP and the immediate receiving waters of Cambridge Bay, Nunavut (NU) (Chaves-Barquero et al., 2016). The issue of antibiotic resistance is of particular importance in the Canadian Territory of Nunavut, which is primarily inhabited by Inuit who practice a number of food harvesting activities (hunting, fishing, shellfish harvesting, etc.) that may create exposure pathways with wastewater constituents including ARBs (Daley et al., 2015, 2017).

To address the current gap in the knowledge of the presence and release of ARGs from arctic WWTPs, the objective of the present study was to evaluate the occurrence of a broad range of clinically relevant ARGs in raw and treated wastewater from wastewater treatment systems in three Arctic Canadian communities (i.e., Clyde River, Pond Inlet and Iqaluit). This study specifically compared the absolute and relative abundance of ARGs in effluent released from WSPs that have long storage times (months) versus a mechanical filtration system with a short hydraulic retention time (minutes). It was also hypothesized that ARG presence and abundance in raw sewage would be greater in Iqaluit, which has a larger population and is the only community in Nunavut that possesses a hospital.

2. Materials and Methods

2.1 Site Descriptions

The three study sites are all located on Baffin Island in the Qikiqtani Region of Nunavut and vary in community size and wastewater treatment processes. Treatment performance assessments were conducted on the WSP systems in Pond Inlet and Clyde River in 2013 and 2014 as part of a large study examining passive wastewater systems in Nunavut. The mechanical WWTP in Iqaluit was sampled in 2015 in order to be able to compare ARG presence in passive

WWTPs operating in small communities to ARG presence in an active WWTP operating in a larger community with a hospital.

2.1.1 Pond Inlet

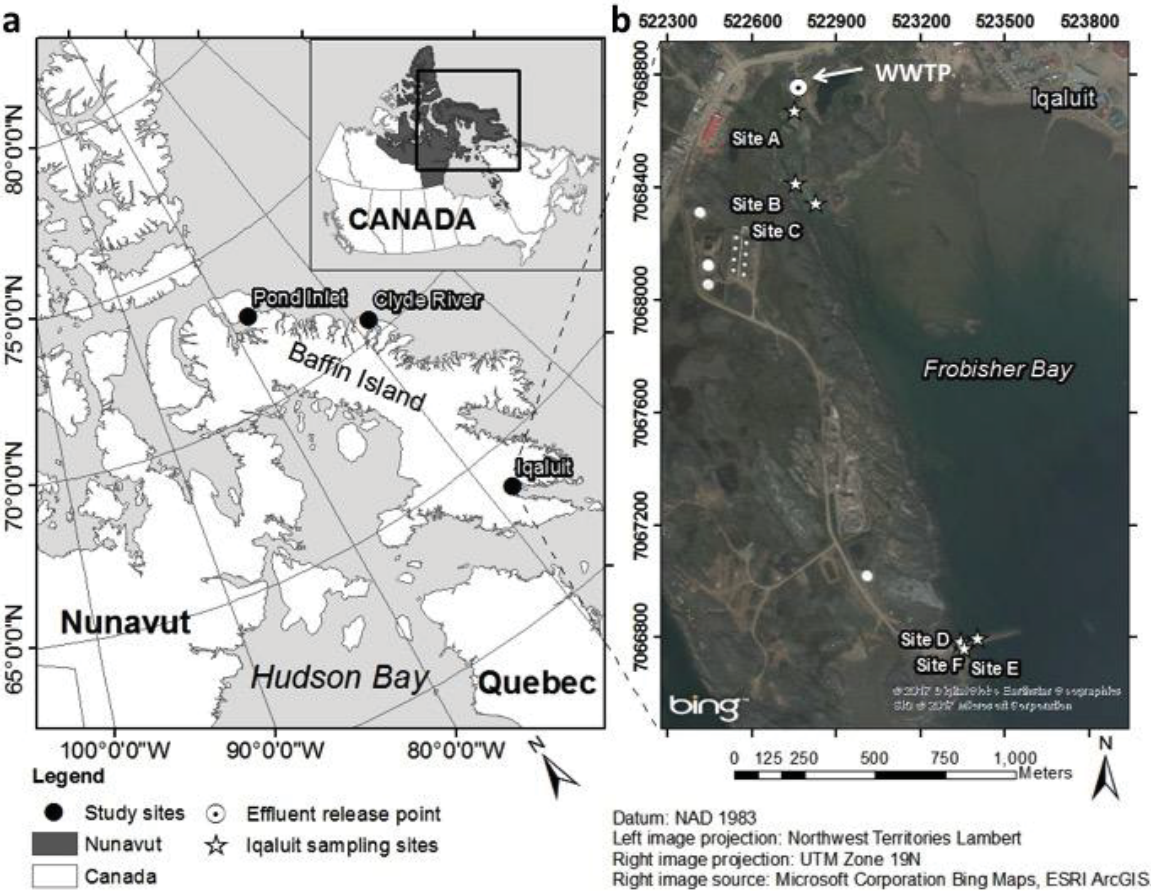
The population of Pond Inlet (72°41'57''N, 77°57'33''W) is approximately 1,673, which results in an expected sewage volume of 6.7×10^7 L per year being delivered to the WWTP by truck (Nunavut Bureau of Statistics, 2014; Wootton et al., 2008). The community's WWTP consists of an engineered WSP, which is decanted once annually in September into the ocean (Eclipse Sound). Samples were taken from the influent (septic trucks), and the effluent (decant samples, regulatory compliance point) in September of 2013 and 2014. Operation of the system was previously described by Ragush et al. (2015) and Huang et al. (2017).

2.1.2 Clyde River

Approximately 983 people live in Clyde River (70°28'26''N, 68°35'10''W) resulting in an expected sewage volume of 3.8×10^7 L per year, which is delivered by truck to the WWTP (Nunavut Bureau of Statistics, 2014; Wootton et al., 2008). The community's WWTP uses two WSPs operated in series (primary and secondary) as the main method of treatment. Samples were taken from the influent (truck) and the effluent (secondary WSP, regulatory compliance point) in June 2013, July 2013, September 2013, June 2014 and September 2014. Additional information about the Clyde River WWTP can be obtained in reports by Ragush et al. (2015) and Huang et al. (2017).

2.1.3 Iqaluit

The capital of Nunavut, Iqaluit (63°44'55''N, 68°31'11''W), has a population of approximately 7,542, leading to an expected sewage volume of 7.2×10^8 L per year (Nunavut Bureau of Statistics, 2014; Wootton et al., 2008). The WWTP also receives sewage from the Qikiqtani General Hospital in Iqaluit. Transport of raw municipal sewage to the WWTP depends on location, whereby 62% of the population have a piped service while 38% have their wastewater removed by truck. The WWTP consists of a mechanical treatment plant that consists of a Salsnes filter that provides solids removal before effluent is continuously decanted into Frobisher's Bay. A small WSP is used for emergency overflows, or for when the plant is not functional. The municipal policy states that any wastewater transported to the WSP is to remain there for one month prior to being decanted into Frobisher's Bay. Samples were taken from the WWTP influent and effluent (regulatory compliance point) twice in September 2015 (during high and low tide events) and once in November 2015. Three receiving water samples from Frobisher's Bay (Figure 1, sites A, B, and C) and three samples from a boat launch (sites D, E, and F) were also taken during high and low tide in September 2015. Sites A, B, and C were chosen based on increasing distance away from the effluent release point to investigate dilution effects. Sites D, E, and F were chosen based on their proximity to local fishing areas. The WWTP was not operational during the September 2015 sampling events, which led to a continuous decant from the WSP without the stipulated one-month holding time. The WWTP was fully functional during the November 2015 sample collection.



160 Figure 1. Map of Nunavut (NU) study communities. a) Location of Pond Inlet, Clyde River and
161 Iqaluit on Baffin Island, NU, Canada; b) Specific Iqaluit receiving water sampling sites. Effluent
162 discharges directly from the WWTP into Frobisher's Bay at the bottom of Koojesse Inlet, NU.
163 Samples were taken at both high and low tides from Sites A-F. Amended from ESRI ArcGIS.

165 *2.2 Assessment of wastewater quality*

All wastewater grab samples were stored at 4 °C, transported to our laboratory facility located at the Nunavut Research Institute (Iqaluit, NU) and analysed within the specific recommended holding times (<24 hours). Each sample was tested for levels of the five-day

Carbonaceous Biochemical Oxygen Demand (CBOD₅), Total Suspended Solids (TSS), ammonia and fecal indicator bacteria (*E. coli* and total coliform bacteria).

Analysis of CBOD₅ was performed using standard 300 mL Wheaton™ BOD bottles in duplicate according to the APHA standard method 5210B (Wootton et al., 2008). TSS analysis was performed using Whatman™ 934-AH 47 mm glass fiber filters (Fisher Scientific) following the APHA standard methods 2540 D (Clesceri et al., 1999). Ammonia was measured using an Orion™ high-performance ammonia electrode (Thermo Scientific) attached to an Orion Star™ series meter (Fisher Scientific), using an ionic strength adjuster (Fisher Scientific), as directed by the manufacturer. Sterile miliQ water was included in all water quality parameter testing as a negative control. During sample collection, *in-situ* measurements of temperature, pH, dissolved oxygen (DO) and electrical conductivity (in Iqaluit) were obtained using a YSI Model 600 sonde (Yellow Springs, OH, USA).

To test for *E. coli* and total coliforms in wastewater samples, the Colilert-18 method was used along with the Quanti-Tray/2000 system, as per manufacturer's instructions (Idexx Laboratories, Lachine, QC, Canada). Following incubation for 18 hours at 35°C, total coliforms and *E. coli* were enumerated by counting the yellow and fluorescent wells (under 365 nm UV light), respectively. All lots of coliform reagents and Quanti-tray/2000 trays were tested to confirm sterility and product performance using triplicate samples and negative (sterile water) controls.

2.3 Molecular methods

Genomic DNA was extracted from samples using MO BIO Powersoil (VWR International, Ville Mont-Royal, QC, Canada) according to manufacturer's specifications.

Briefly, 30 – 500 mL of sample was either filtered using a Millipore Vacuum Manifold, Microfil Filtration funnels, and S-Pak 0.45 µm pore size membranes (Millipore, Inc., Bedford, MA), or centrifuged at 3200 × *g* for 10 min, depending on the turbidity of the sample. The entire filter membrane or cell pellet was inserted into a PowerBead tube and subsequently used for DNA extraction. Sterile water samples were also filtered on to membranes and used as negative controls.

Antibiotic resistance gene copy numbers were detected through quantitative real-time Taqman PCR (Taqman qPCR). Primers and probes used to detect a class I integrase gene and various ARGs were obtained from the literature (Barraud et al., 2010; Böckelmann et al., 2009; Colomer-Lluch et al., 2011; Colomer-Lluch et al., 2014; Czekalski et al., 2012; Francois et al., 2003; Heurer et al., 2008; Lachmayr et al., 2008; Suzuki et al., 2000) and detailed in Supporting Material (Table S1). The following targets were measured in the study: class I integrase *int1*; class A β-lactamase genes *bla_{CTX-M}* and *bla_{TEM}*; macrolide-lincosamide-streptogramin type B resistance gene *erm(B)*; fluoroquinolone resistance gene *qnr(S)*; sulphonamide resistance genes *sul1* and *sul2*; tetracycline resistance gene *tet(O)*; and the methicillin resistance gene *mecA*.

Control plasmids for *int1*, *bla_{TEM}*, *sul1*, and *sul2* were obtained from Dr. E. Topp (University of Western Ontario, London, ON, Canada) and described in Rahube et al. (2014). For targets *bla_{TEM}*, *erm(B)*, and *qnr(S)*, products were amplified from northern WWTP environmental genomic DNA (gDNA) samples using forward and reverse primers listed in Table S1. PCR products were cloned into the plasmid pCR[®]2.1-TOPO by using the TOPO[®] PCR cloning kit (ThermoFisher Scientific). The PCR consisted of 1x Hot Start Taq 2x Master Mix (NEB), primers at 0.2 µM, and 1 µl of template in a 25 µl reaction. PCR conditions were 95°C for 30 s; 30 cycles of 95°C for 30 s, annealing at 56°C for *bla_{TEM}* and 63°C for *erm(B)* and

qnr(S)) for 1 min, extension at 72°C for 1 min/kbp; and a final extension at 72°C for 5 min. The last control plasmid was constructed for *tet(O)* and *mecA* using a gBlock Gene Fragment (Integrated DNA Technologies, Toronto, ON, Canada) consisting of both target sequences. The synthesized double stranded sequence was cloned into the plasmid pJET1.2/blunt using the CloneJET PCR Cloning Kit as per the manufacturer's specifications (ThermoFisher Scientific). All plasmid controls were confirmed as harbouring the desired ARG target inserts through DNA sequencing (Génome Québec, QC, Canada). Plasmid constructs were transformed into One Shot® TOP10 chemically competent *E. coli* (ThermoFisher Scientific). *E. coli* cultures containing positive plasmid controls were inoculated in 5 mL Luria-Bertani (LB) medium (Sambrook et al., 1989) supplemented with ampicillin (100 µg/mL) and cultured overnight at 37°C with agitation (200 rpm). All plasmids were extracted using the GenElute™ Plasmid Miniprep Kit (Sigma Aldrich, Oakville, ON, Canada) and used for the production of qPCR standard curves following the determination of the plasmid DNA concentration using Quant-iT PicoGreen dsDNA Assay Kit (ThermoFisher Scientific). All standard qPCR runs included negative controls using laboratory grade water as template. Limit of detection was 5 copies per qPCR reaction (McConnell, 2017), corresponding to 1 and 16.7 ARG copies/mL for 500 and 30 mL sample volumes, respectively.

The TaqMan qPCR reaction mixture consisted of 1× Sso Advanced™ Universal Probes Supermix (Bio-Rad), primers at 0.9 μM, TaqMan probe at 0.25 μM and 1 μl of template DNA for a final volume of 20 μl. Reaction conditions were 3 min at 95°C; 40 cycles of 15 s at 95°C and 30 s at 62°C with reaction being performed on a Bio-Rad CFX96 Touch system (Hercules, CA, USA). To quantify universal 16s rRNA gene fragments, SYBR Green qPCR was used (Table S1). The reaction mixture consisted of 1× Power SYBR® Green PCR Master Mix

(ThermoFisher Scientific), primers at 0.4 μ M and 1 μ l of template DNA for a final volume of 20 μ l. Reaction conditions were 10 min at 95°C; 40 cycles of 15 s at 95°C, 30 s at 55°C, and 30 s at 72°C; concluding with a melt curve of 30 s at 55°C increasing by 0.5°C each cycle until 95°C. All qPCR reactions were run in triplicate, along with negative controls without template DNA. The absence of PCR inhibitors was confirmed in spiking experiments as detailed by Huang et al. (2017).

2.4 Data Analysis

To account for between-run variation, raw data were exported from the Bio-Rad qPCR system and imported into the Factor-qPCR program (Ruijter et al. 2015). The program automatically determined the fluorescence threshold for all samples and calculated the individual threshold cycles and the efficiency values. The results were exported, and the mean sample efficiency was calculated as the arithmetic mean of all replicates, excluding any replicates exhibiting a 5% difference in mean efficiency. To account for differences in efficiencies, a one-point calibration (OPC) method for absolute quantification was used (Brankatschk et al., 2012). For relative quantification, the gene copy number of each individual gene was then normalized to the gene copy number of the 16S rRNA gene in a given sample and log transformed ($\log(\text{gene copies}/16\text{S rRNA gene copies})$). For absolute quantification, samples were normalized to the initial water volume used for gDNA extraction to generate gene copies per mL of water. One-way analysis of variance (ANOVA), Tukey HSD test, and t-tests (SPSS Statistics for Mac; IBM Corp., Armonk, NY) were used to assess statistically significant differences ($P < 0.05$). The effect of treatment on sample ARG profiles was also submitted to a principal component analysis

using the on-line freeware ClustVis (Metsalu and Vilo, 2015; accessible at <http://biit.cs.ut.ee/clustvis/>).

3. Results

3.1 Characteristics of raw and treated wastewater

CBOD₅, TSS, ammonia, *E. coli*, and coliforms were measured to compare water quality across all three WWTPs (Table 1). WSER values and other relevant guidelines were also included in Table 1 to compare how the WWTPs were performing with respect to the Canadian regulations. With the exception of ammonia levels, all values were higher than the specified WSER values (Table 1). The effluent water from the Iqaluit WWTP consistently exhibited the highest values for CBOD₅, TSS, *E. coli* and total coliforms, followed by Pond Inlet and lastly by Clyde River. Overall, the reduction in nutrients and fecal indicator bacteria was greater in the WSP-based treatment systems compared to the mechanical system in Iqaluit, likely due to the longer retention times in the ponds (Table 1).

The wastewater in Iqaluit had a temperature of 15 °C, pH of 7.4, DO of 4.7 mg/L, and electrical conductivity of 408 µS/cm. The receiving water in Frobisher Bay was measured to a temperature of 1.9 °C, pH of 7.9, DO of 10.8 mg/L, and electrical conductivity of 38,161 µS/cm. In Pond Inlet and Clyde River, the WSP temperature ranged from 7-11°C, pH of 7.5 and DO from <0.2 to 1.3 mg/L during the treatment seasons of 2013 and 2014 (Ragush et al., 2015).

Table 1. Comparison of the wastewater effluent quality in Pond Inlet (WSP), Clyde River (WSP), and Iqaluit (mechanical) with guideline values set by the Canadian Wastewater Systems Effluent Regulations (WSER). Average values are shown with standard deviations (n-1).

| Water quality parameter | WSER | Clyde River ^c n=5 | Pond Inlet ^d n=5 | Iqaluit ^e n=3 |
|-----------------------------------|-------------------|---------------------------------|---------------------------------|----------------------------------|
| CBOD ₅ (mg/L) | 25 | 82 ± 24 (68%) ^f | 109 ± 7 (50%) ^f | 269 ± 6 (5%) ^f |
| TSS (mg/L) | 25 | 30 ± 16 (44%) ^f | 71 ± 9 (-54%) ^f | 232 ± 12 (-21%) ^f |
| Ammonia (Unionized) (mg/L-N) | 1.25 | 0.21 ± 0.11 (-40%) ^f | 0.40 ± 0.08 (0%) ^f | 0.03 ± 0.01 (-200%) ^f |
| <i>E. coli</i> (log(MPN/100 mL)) | 2.30 ^a | 4.97 ± 0.36 (1.32) ^g | 5.84 ± 0.07 (1.74) ^g | 6.89 ± 0.37 (0.06) ^g |
| Total Coliforms (log(MPN/100 mL)) | 3.70 ^b | 6.88 ± 0.96 (1.12) ^g | 6.96 ± 0.06 (2.08) ^g | 7.49 ± 0.12 (0.31) ^g |

a: Atlantic Canada Wastewater Guidelines for Collection, Treatment, and Disposal from 2006

b: Newfoundland and Laborator Regulation 65/03: Environmental Control Water and Sewage Regulations, 2003 under the Water Resources Act.

c: Secondary cell treatment quality from 2013-2014

d: Effluent quality from 2013-2014

e: Effluent quality from 2015

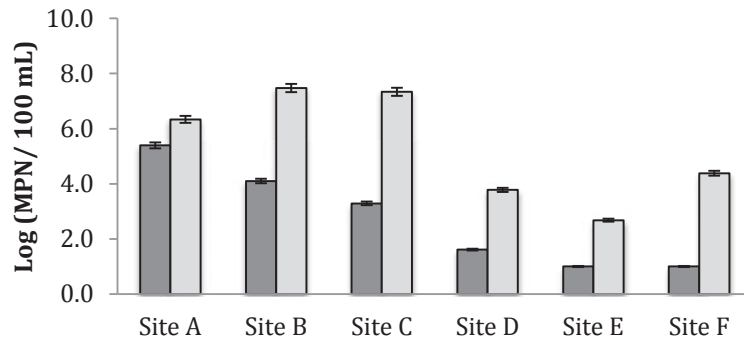
f: Percent decrease from influent values of CBOD₅, TSS and ammonia is included in parentheses (negative values represent an increase).

g: Log removal (influent-effluent) is included in parentheses, all removal differences were significant (P < 0.05) except for Iqaluit *E. coli*.

3.2 Fecal indicator bacteria in wastewater and receiving waters in Iqaluit

Regardless of whether the Iqaluit WWTP was functional or not, the mechanical treatment yielded no significant difference in *E. coli* counts between influent and effluent samples, while there was a slight but significantly (P < 0.05) higher total coliform count in the influent when compared to the effluent (Table 1). At low tide, all receiving water samples obtained from Frobisher's Bay (Iqaluit) harboured significantly (P < 0.05) higher counts of total coliforms (Site A: 1-log, Site B: 3-log, Site C: 4-log, Site D: 2-log, Site E: 2-log, Site f: 3-log) and *E. coli* (Site A: 0.5-log, Site B: 4-log, Site C: 5-log, Site D: 2-log, Site E: 1-log, Site F: 3-log) when compared to high tide (Figure 2a-b).

(a) Total coliforms



(b) *E. coli*

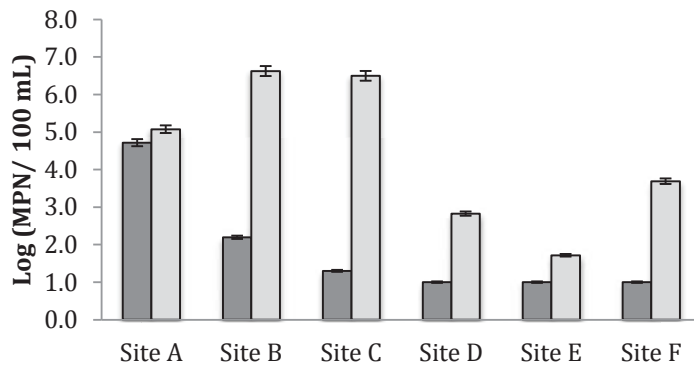


Figure 2. Average most probable number (MPN) content of fecal indicator bacteria, (a) total coliforms and (b) *E. coli*, in water at different sites (Figure 1) in Frobisher's Bay close to outlet of the Iqaluit WWTP. The dark and light grey bars represent high and low tide, respectively. Error bars represent the standard deviation (n-1).

3.3 Abundance of antibiotic resistance genes in raw and treated wastewater

The WSP systems significantly ($P < 0.05$) reduced the sum of all ARGs (copies/mL) detected in the influent in Clyde River (449 copies/mL) and Pond Inlet (383 copies/mL) to effluent contents of 290 and 205 copies/mL, respectively. In contrast, the mechanical system in

Iqaluit affected no significant differences ($P > 0.05$) between the influent (sum of all ARGs 279 copies/mL) and the effluent (271 copies/mL) water samples. Looking at the content of individual ARGs, no differences ($P > 0.05$) were observed between in the raw and treated wastewater in Iqaluit (Figure 3a). Effluent water samples in Clyde River and Pond Inlet contained between 6 and 70 copies per mL of individual ARGs (Figures 3b-c). When comparing influent and effluent samples from the WWTP in Clyde River, significantly lower ($P < 0.05$) absolute abundances (range of 9-127 gene copies/mL) of *int1*, *bla_{TEM}*, *sul1*, *tet(O)*, and *erm(B)* were observed in the effluent (Figure 3b). The ARG content in effluent samples from Pond Inlet's WWTP were also significantly lower ($P < 0.05$) with absolute abundances (range of 7-95 gene copies/mL) of *int1*, *bla_{TEM}*, *sul1*, *sul2*, *tet(O)*, and *erm(B)* being lower in the effluent, while *qnr(S)* gene copies per mL increased significantly ($P < 0.05$) from 7.7 to 13.3 gene copies/mL in the influent and effluent, respectively (Figure 3c). The 16S rRNA gene copy numbers in the influent dropped by less than 1-log in the effluent water samples, illustrating the WWTPs affecting a slight decrease in the overall number of bacteria being released into the environment (Figure 3a-c). Interestingly, 16S rRNA bacterial populations in effluent samples were greater in Iqaluit (4×10^8 copies/mL) than in Clyde River (2×10^7 copies/mL) and Pond Inlet (3×10^7 copies/mL).

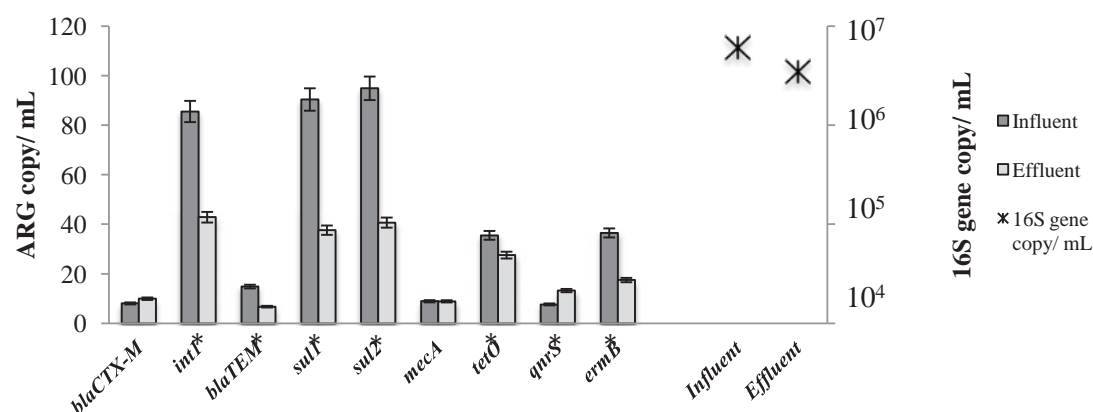
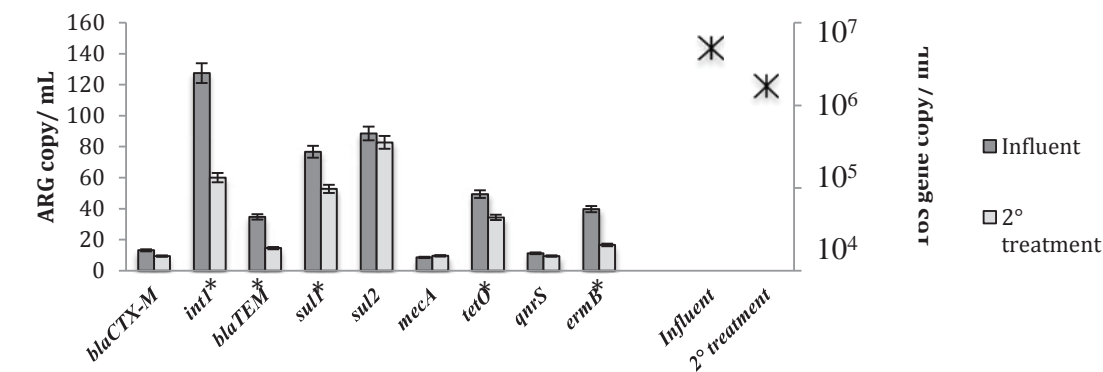
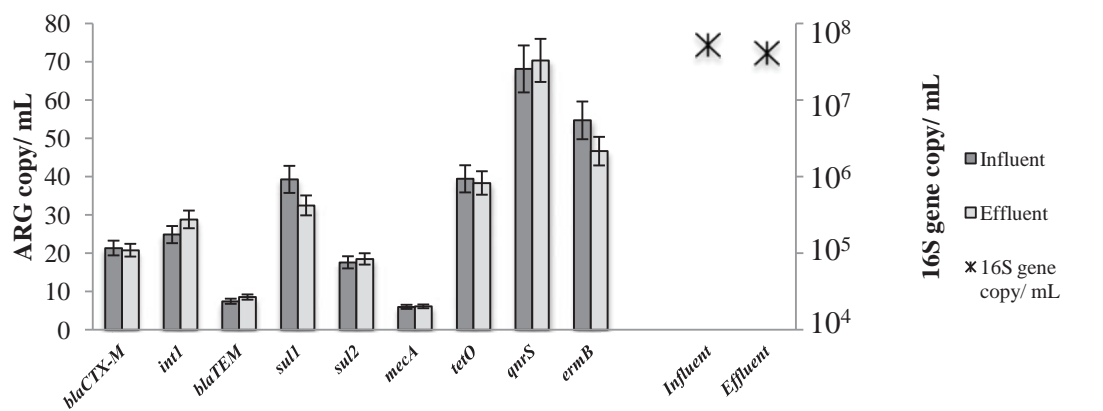


Figure 3. Average absolute abundance of ARGs. (a) Iqaluit's WWTP influent (n=3) and effluent (n=3) in September and November of 2015. No significant differences ($P > 0.05$) were observed

for influent and effluent samples or across samples obtained on September and November sampling dates, despite the difference in functional state of the WWTP. (b) Clyde River's WWTP influent (n=5) and effluent (secondary WSP, n=5) in June 2013, July 2013, September 2013, June 2014 and September 2014. No significant ($P > 0.05$) seasonal or annual differences were observed. (c) Pond Inlet's WWTP influent (n=4) and effluent (decant samples, n=4) in September 2013 and September 2014. Error bars represent standard deviation. Significant differences ($P < 0.05$) for ARG content in raw and treated wastewater in (b) and (c) are indicated by an asterisk (*).

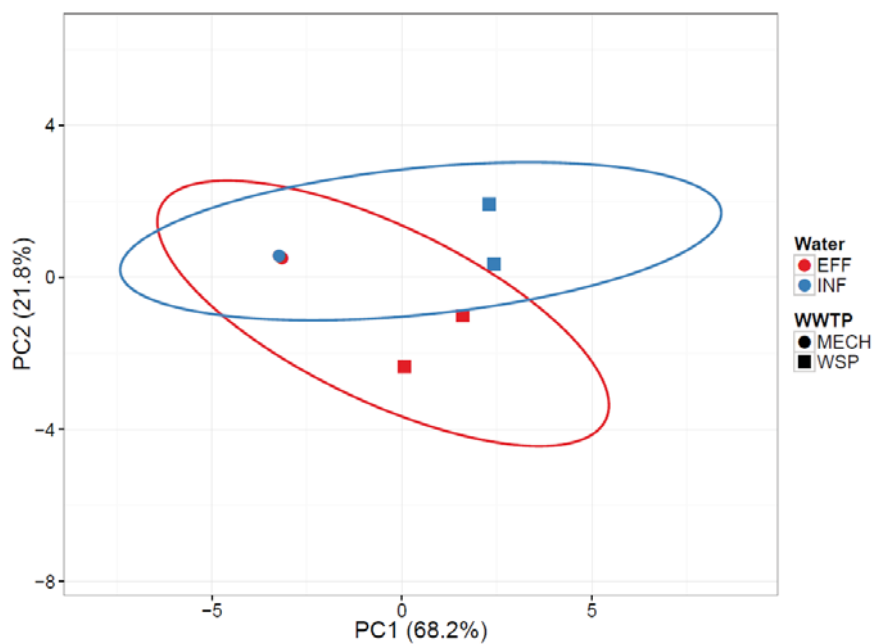
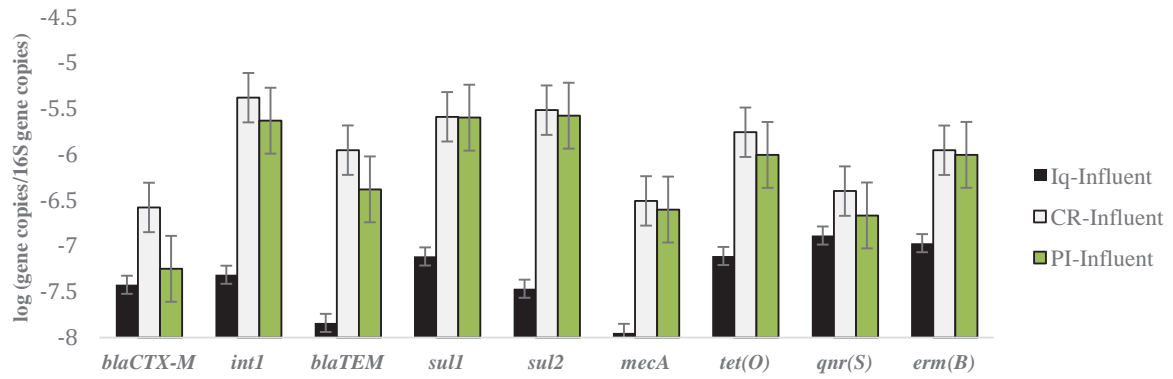


Figure 4. Principal component analysis of the ARG profile in influent and effluent wastewater samples from the mechanical treatment plant in Iqaluit and the WSP systems in Clyde River and Pond Inlet.

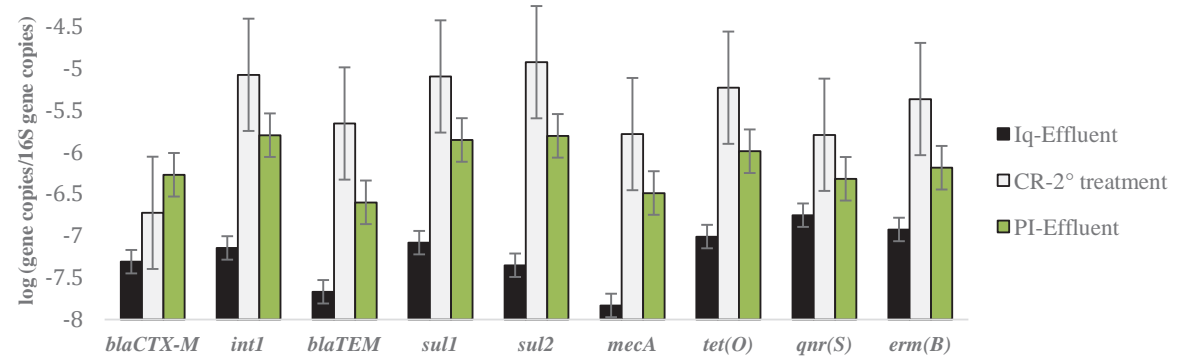
Principal component analysis showed that WSP treatment in Clyde River and Pond Inlet caused a change in the ARG profile, i.e. the proportion of each ARG in a given sample, between influent and effluent samples while the mechanical treatment in Iqaluit did not (Figure 4). Moreover, the treatment method and total ARG concentration affected the effluent ARG profile, which also differed among the communities (Supplementary Material Figures S1 and S2).

The relative abundance of the ARGs, a measure that expresses the frequency of an ARG in the total bacterial population (here measured as 16S rRNA GCs), also varied across the three WWTPs (Figure 5). Iqaluit influent samples contain significantly ($P < 0.05$) lower relative abundance levels of *int1*, *bla_{TEM}*, *sul1*, *sul2*, *mecA*, *tet(O)*, and *erm(B)*, by up to 2 orders of magnitude, when compared to Clyde River and Pond Inlet influent samples (Figure 5a). The differences increase when comparing effluent samples. The effluent in Iqaluit harboured significantly ($P < 0.05$) lower levels of all ARGs (except for *bla_{CTX-M}* in Clyde River) as compared to the effluent in Clyde River and Pond Inlet (Figure 5b).

(a)



(b)



| | <i>blaCTX-M</i> | <i>int1</i> | <i>blaTEM</i> | <i>sul1</i> | <i>sul2</i> | <i>mecA</i> | <i>tet(O)</i> | <i>qnr(S)</i> | <i>erm(B)</i> |
|-----------|-----------------|-------------|---------------|-------------|-------------|-------------|---------------|---------------|---------------|
| Iq vs. CR | NS | P=0.0009 | P=0.0016 | P=0.0016 | P=0.0004 | P=0.0055 | P=0.0022 | P=0.014 | P=0.0146 |
| Iq vs. PI | NS | P=0.0014 | P=0.0054 | P=0.0022 | P=0.0006 | P=0.0025 | P=0.0047 | P=0.0387 | P=0.0095 |
| CR vs. PI | NS | NS | P=0.0500 | NS | NS | NS | NS | NS | NS |

Figure 5. Comparison of the average relative abundance of ARGs from either influent (a) or effluent (b) WWTP samples from Iqaluit (Iq), Clyde River (CR), and Pond Inlet (PI). Statistical significance from ANOVA and Tukey's HSD test are included for both influent and effluent samples, below each graph. NS = nonsignificant.

Comparison of the relative abundance in influent and effluent samples within each community revealed that the relative abundance of *sul1*, *sul2*, *mecA*, *tet(O)* and *qnr(S)* were significantly ($P < 0.05$) higher in samples from the secondary WSP in Clyde River than in the incoming wastewater. Similar enrichments in relative abundance of ARGs were not observed ($P > 0.05$) in the other two communities.

3.4 Tides and abundance of antibiotic resistance genes in receiving waters

To examine the effect of tidal changes on the ARG abundance in the Iqaluit receiving waters, the presence of the nine target genes were tested in both high and low tide receiving water samples from Frobisher's Bay near the Iqaluit WWTP. The relative abundances varied by site and by gene, as demonstrated in Figure 6. Site A (closest to the outlet from the WWTP) exhibited no significant difference in the relative levels of ARGs when comparing high and low tide samples (Figure 6). However, the other 5 sites showed significantly ($P < 0.05$) higher relative abundances of several ARGs in the receiving water samples during low tide as compared to high tide samples: Site B (*mecA*, *qnr(S)*), Site C (all nine targets), Site D (all nine targets), Site E (all targets except for *erm(B)*), and Site F (*bla_{CTX-M}*, *bla_{TEM}*, *mecA*, *tet(O)*). Additionally, when sites A, B, and C are clustered and compared to sites D, E, and F, the sites DEF have significantly ($P < 0.05$) higher relative ARG abundance than sites ABC.

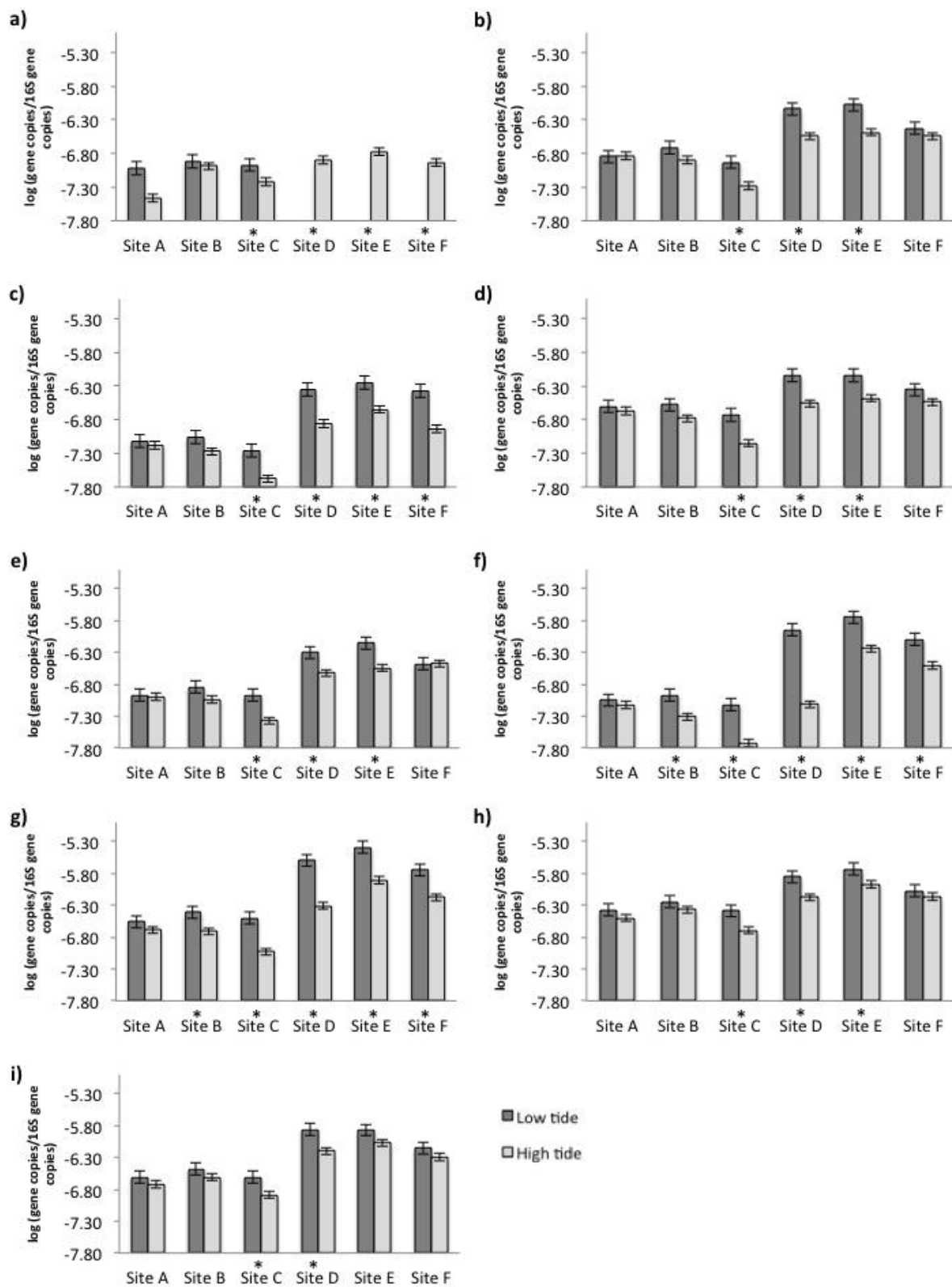


Figure 6. Relative abundance of ARGs in high and low tide receiving water samples (n=3) in Frobisher's Bay, Iqaluit, NU. a) *bla_{CTX-M}*; b) *int1*; c) *bla_{TEM}*; d) *sul*; e) *sul2*; f) *mecA*; g) *tet(O)*; h) *qnr(S)*; i) *erm(B)*. Absence of a value indicates the target was below the detection limit (LOD). Significant differences ($P < 0.05$) between low and high tide on each site are indicated by an asterisk (*).

4. Discussion

In this study, three Arctic Canadian wastewater treatment facilities were investigated for both water quality and the presence of antibiotic resistant genes in raw and treated wastewater. Biological and chemical reaction rates are highly influenced by temperature, and given that the air temperature often remains below 10°C during the summer in these northern communities, these WWTPs present an interesting comparison to similar systems in southern Canada (Holeton et al., 2011; Ragush et al., 2015). Measurements of wastewater quality parameters showed that current systems generally provide primary treatment only and would not comply with the WSER that is implemented in southern Canada. The WSP systems in Pond Inlet and Clyde River provided a greater level of treatment than the mechanical plant in Iqaluit, indicating that some biological treatment is occurring in WSPs in this region.

Raw and treated wastewater samples taken from Iqaluit, Pond Inlet, and Clyde River were all found to contain different absolute abundances and diversities of ARGs. Tundra wetlands are also commonly used in many Canadian Arctic communities for municipal wastewater treatment, and recent studies have shown that they can be effective in reducing concentrations of conventional wastewater pollutants (Yates et al., 2012; Hayward et al., 2014). The ability of these systems to remove emerging contaminants, such as ARGs, warrants further

investigation. It should also be noted that in Pond Inlet and Clyde River wastewater is transported from individual homes to the WSP using septic haulage trucks. Each truck would contain wastewater from several households. Several samples were collected from different trucks as they discharged their contents into the WSP, but there is uncertainty associated with how well these sub-samples of raw sewage represent the entire community.

Treatment in WSP-based systems altered the ARG profile and caused a reduction in the absolute number of *E. coli*, ARGs as well as the 16S rRNA GCs, indicating that WSP treatment reduced the total bacterial counts, including ARG containing bacteria in the effluent. However, the mechanical system in Iqaluit had no effect on the effluent ARG profile, *E. coli*, ARG or bacterial (16S rRNA GC) loads exiting the WWTP, indicating a lack of microbial removal.

Overall ARG levels in the effluent from the three Baffin Island treatment facilities were lower at ~2 log copies/ml compared to ≥ 4 log copies/ml in the effluent from the WSP in Cambridge Bay located on Victoria Island, Nunavut (Chaves-Barquero et al., 2016) and southern Canadian WWTPs with a hospital influence (McConnell, 2017; Narciso-da-Rocha et al., 2014; Volkmann et al., 2004). Based on previously observed antimicrobial dispensing rates, Nunavut remains on the lowest spectrum observing 5-5.99 defined daily doses (DDDs) per inhabitant as compared to a national average of 6.5 DDDs (Canadian Antimicrobial Resistance Surveillance System Report, 2016). Having less selective pressure entering the wastewater system could be one explanation as to why lower overall ARG levels were observed in the Qikiqtani region of Nunavut compared to the Southern WWTPs (McConnell, 2017; Narciso-da-Rocha et al., 2014; Volkmann et al., 2004). Cambridge Bay, where “Southern” ARG levels were found (Chaves-Barquero et al., 2016), may be a special case due to the presence of the Canadian High Arctic

Research Station and influx of people from areas with higher antibiotic prescription rates and/or natural/climatic differences between the Qikiqtani and Kitikmeot regions in Nunavut.

Interestingly, for the Clyde River WSP system, even though absolute ARG abundance decreased throughout the treatment continuum, effluent samples were found to contain significantly ($P < 0.05$) higher relative abundances of multiple ARGs (i.e., *sul1*, *sul2*, *mecA*, *tet(O)*, and *qnr(S)*) by the end of the treatment process, when compared to the influent levels indicating that these ARGs were enriched in the overall microbial community during the lengthy holding time in the secondary WSP. Possible explanations for this observation may be that antibiotics (especially fluoroquinolones, trimethoprim, and sulphonamides) are poorly removed during primary treatment processes (Chaves-Barquero et al., 2016; Göbel et al., 2005; Lindberg et al., 2006; Nakata et al., 2005). This antibiotic presence could be placing a selective pressure on bacteria within the wastewater system, leading to the enrichment of resistant bacteria and subsequent release into the receiving waters (Szczepanowski et al., 2009). The most recent antimicrobial surveillance report indicates that antibiotics, including amoxicillin and cephalexin (both beta-lactams), azithromycin (macrolide), doxycycline (tetracycline), ciprofloxacin (fluoroquinolone), trimethoprim and sulfamethoxazole (sulphonamides), are being widely used in the Northern Territories (Canadian Antimicrobial Resistance Surveillance System Report, 2016). Future efforts will investigate the presence of antibiotics in the wastewater system to analyse their influence on the ARG presence.

A similar enrichment in ARGs was not observed in Pond Inlet's WSP. This difference could be due to the higher initial ARG load in Clyde River influent wastewater and also the fact that in Clyde River, the decant does not always occur yearly due to their increased storage capability. Therefore, there may be more available ARGs and time for vertical and horizontal

gene transfer to occur within the bacterial communities found in that WSP. This may explain the increase in relative abundance in the Clyde River WSP versus the Pond Inlet WSP as well as the mechanical system in Iqaluit with its short retention time. Taken together there appears to be a trade-off whereby increased initial ARG load and/or retention time in the wastewater treatment process improves organic material removal, but given the right circumstances may cause an enrichment of ARB and ARGs.

It is also worth noting that the relative ARG abundance levels in the raw sewage coming from Iqaluit were lower than in both Pond Inlet and Clyde River, despite a much larger population in Iqaluit. This may be due to differences in the infrastructure among the communities where Pond Inlet and Clyde River are serviced 100% by water and sewage trucks while in Iqaluit about 60% of customer connections are on piped services. Previous research has shown that trucked services markedly reduce water consumption (Daley et al., 2015; Heinke et al., 1991), which in turn could increase the proportion of fecal matter including ARB and ARGs in the raw sewage. Future research involving antibiotic residue analysis of wastewater samples may help to provide insight into overall antibiotic pressure that is present in these wastewater systems, and elucidate mechanistic causes of the different ARG profiles observed in raw and treatment wastewater from the three communities.

When investigating differences between high and low tide ARG abundances in the receiving waters in Iqaluit, it was expected that a higher abundance would be seen at low tide, due to a dilution effect occurring at high tide. Sites A and B located closer to the WWTP were less diluted, therefore it was not unexpected to see small differences between high and low tide sampling. However, it was surprising to see an increase in relative ARG abundance at Sites D, E, and F, due to their distance from the WWTP's discharge point in Koojesse Inlet and increasing

water depths. One explanation could be the presence of the boat launch, which extends out from the natural shoreline, and may act as a flow barrier leading to increased sedimentation. This may promote enrichment of ARGs in the sediment, which could be released during the tidal cycles and weather events. The wave action was high during the low tide sampling, which may have promoted the release of ARGs from the sediment. Given these data and the drastic tidal change in Iqaluit (e.g. parts of Frobisher's Bay flow being exposed at low tide, with a daily tidal variance of about 8 – 12 m) (Environment Canada, 2016), it may be warranted to quantify the presence of ARGs in the sediment in the receiving waters of Iqaluit.

5. Conclusions

This study provides evidence of the presence of low levels of multiple ARGs entering and leaving three municipal WWTPs, which represent common wastewater treatment systems in the Canadian Arctic. Overall, WSPs had better ARG removal compared to the mechanical system. However, an enrichment of the relative ARG abundance was observed in one WSP with longer holding times, illustrating the potential impact of such wastewater treatment systems on the ARG enrichment processes. The data from this study provides the foundation for future research efforts that focus on developing a complete understanding of the risk posed to the indigenous populations who may be hunting and fishing in receiving waters.

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We would like to extend our gratitude to the community members of Iqaluit, Clyde River, and Pond Inlet. We would also like to thank our team members (Joanna Poltarowicz, Kiley Daley, Jenny Hayward, Audrey Hiscock, and Mandy McConnell) for their field and laboratory

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Table S1. Quantitative PCR primer and probe sequences.

Supplementary data

| Target gene | Primers/ probes | Sequences | References |
|-----------------------|--|--|---|
| 16S rRNA ^a | 1369F 1492R | CGGTGAATACGTTTCYCGG GGWTACCTTGTTACGACTT | Suzuki et al., 2000 |
| erm(B) | ermBF ermBR ermBP | GGATTCTACAAGCGTACCTTGGA GCTGGCAGCTTAAGCAATTGCT FAM-CACTAGGGTTGCTCTTGACACTCAAGTC- BHQ1 | Böckelmann et al., 2009 |
| mecA | mecAF mecAR mecAP | CATTGATCGCAACGTTCAATTTAAT TGGTCTTTCTGCATTCTCTGGA FAM-CTATGATCCCAATCTAACTCCACATACC- BHQ1 | Böckelmann et al., 2009, Francois et al., 2003 |
| tet(O) | tetOF tetOR tetOP | AAGAAAACAGGAGATTCCAAAACG CGAGTCCCCAGATTGTTTTTAGC FAM-ACGTTATTTCCTGTTTATCACGGAAGCG- BHQ1 | Böckelmann et al., 2009 |
| blaCTX-M | CTX-M UP CTX-M LP CTX-M probe | ACCAACGATATCGCGGTGAT ACATCGCGACGGCTTTCT FAM-TCGTGCGCCGCTG-BHQ1 | Colomer- Lluch et al., 2011 |
| blaTEM | TEM UP TEM LP TEM probe | CACTATTCTCAGAATGACTTGGT TGCATAATTCTCTTACTGTCATG FAM-CCAGTCACAGAAAAGCATCTTACGG-BHQ1 | Lachmayr et al., 2008 |
| sul1 | qSUL653f qSUL719r tpSUL1 | CCGTTGGCCTTCCTGTAAAG TTGCCGATCGCGTGAAGT FAM -CAGCGAGCCTTGCGGCGG-BHQ1 | Czekalski et al., 2012, Heurer et al., 2008 |
| sul2 | qSUL2_595f qSUL2_654r tpSUL2_614 | CGGCTGCGCTTCGATT CGCGCGCAGAAAGGATT FAM -CGGTGCTTCTGTCTGTTTCGCGC-BHQ1 | Czekalski et al., 2012, Heurer et al., 2008 |
| qnr(S) | qnrS UP qnrS LP qnrS probe | CGACGTGCTAACTTGCGTGA GGCATTGTTGGAACTTGCA FAM-AGTTCATTGAACAGGGTGA-BHQ1 | Colomer- Lluch et al., 2014 |
| int1 | intI1-LC1 intI1-LC5 intI1-probe | GCCTTGATGTTACCCGAGAG GATCGGTCGAATGCGTGT FAM-ATTCCTGGCCGTGGTTCTGGGTTTT-BHQ1 | Barraud et al., 2010 |

^aqPCR assay conditions for 16S rRNA, 95°C for 10 min; 40 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s. For all the antibiotic resistance related target genes the following qPCR assay conditions were used: 95°C for 3 min; 40 cycles of 95°C for 15 s, 62°C for 30 s.

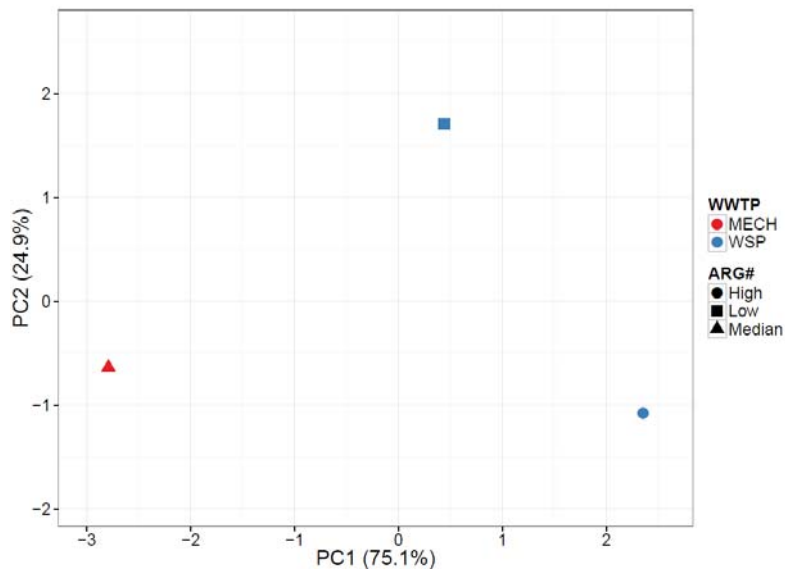


Figure S1. Principal component analysis of the ARG profile (9 markers) in the effluent water from Iqaluit (red triangle), Clyde River (blue circle) and Pond Inlet (blue square).

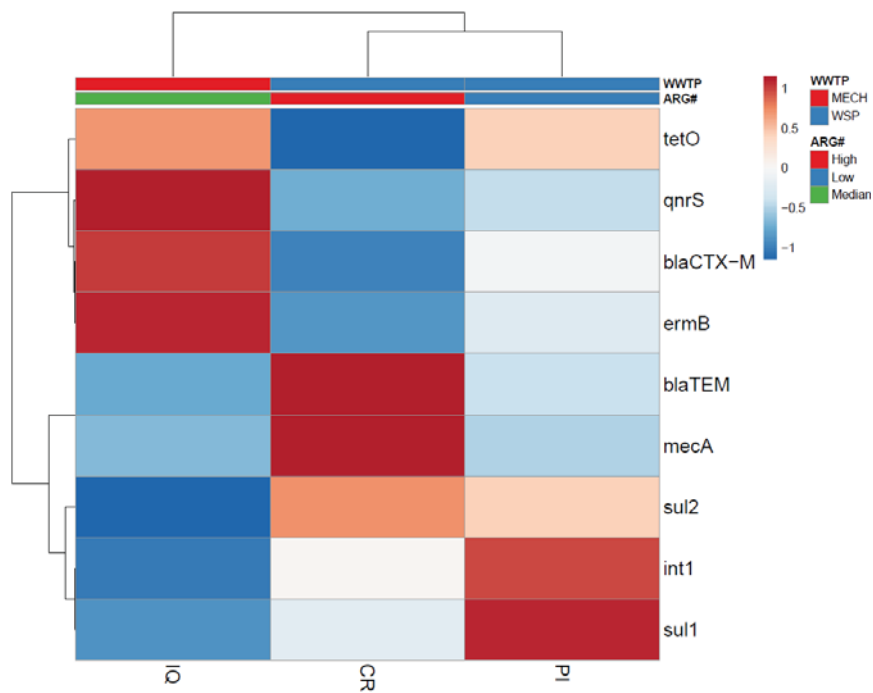
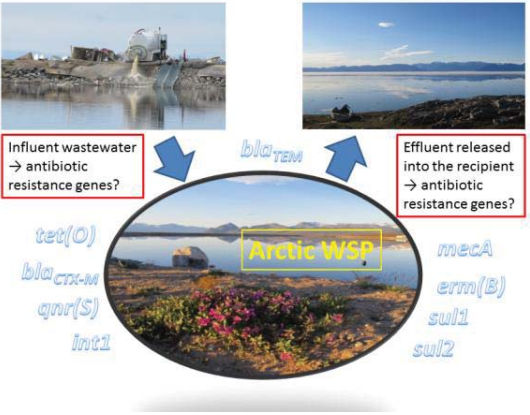


Figure S2. Heatmap of the ARG markers in the effluent water from Iqaluit, Clyde River and Pond Inlet.

673 Graphical abstract for the Table of Contents only



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